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Multifunctional Bioactive Glass and Glass-Ceramic Biomaterials with Antibacterial Properties for Repair and Regeneration of Bone Tissue

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Abstract

Bioactive glasses (BGs) and related glass-ceramic biomaterials have been used in bone tissue repair for over 30 years. Previous work in this field was comprehensively reviewed including by their inventor Larry Hench, and the key features and properties of BGs are well understood. More recently, attention has focused on their modification to further enhance the osteogenic behaviour, or further compositional changes that may introduce additional properties such as antimicrobial activity. Evidence is emerging that BGs and related glass-ceramics may be modified in such a way as to simultaneously introduce more than one desirable property. The aim of this review is therefore to consider the evidence that these more recent inorganic modifications to glass and glass-ceramic biomaterials are effective, and whether or not these new compositions represent sufficiently versatile systems to underpin the development of a new generation of truly multifunctional biomaterials to address pressing clinical needs in orthopaedic and dental surgery. Indeed, a number of classical glass compositions exhibited antimicrobial activity, however the structural design and the addition of specific ions, i.e. Ag^{+}, Cu^{+}, and Sr^{2+}, increased the antimicrobial activity.

1. Introduction

Multiple degenerative and inflammatory joint and bone diseases affect millions of people worldwide. In fact, in 2007 the Bone and Joint Decade's association predicted that the percentage of people over 50 years of age affected by bone diseases will double by 2020 [1, 2]. The huge increase in joint and bone implant surgeries parallels that of medical-device associated infections (MDAIs) [3-7]. Bacterial infections associated with contamination of implanted medical devices are a critical complication that often leads to the failure of the implant with significant impact concerning public health in developed countries [8-10]. Moreover, the management of MDAIs often requires the need for surgical intervention or/and prolonged usage of intravenous or oral antibiotic therapies leading to bone loss and significant morbidity resulting in severe limitations to the patients regarding normal life and wellbeing [7, 11, 12].

To summarise, there is now a pressing clinical need to develop innovative biomaterials or device surfaces that provide the dual functionality of both: bone tissue regeneration and inhibition of pathogenic microorganisms. Such a technology would contribute significantly to a surgical solution to the problem of increasing infection rates in the most vulnerable patient groups. Extensive research have led to the development of bioactive glasses (BGs) and related glass-ceramics with excellent biocompatibility and bioactivity [2, 13-20]. However, clinical applications have so far been limited to what bone bonding and integration concerns. Their range of uses can be extended significantly by a better understanding of the structural role of each

component in the glass, allowing intelligent design of the glass and glass-ceramics and thus introducing multifunctionality.

The purpose of this review is to determine whether or not BGs and related glass-ceramic biomaterials have the potential to provide the first generation of multifunctional biomaterials for the manufacture of advanced medical devices for bone surgery in orthopaedic and dental surgery. In addition, the authors will consider how inorganic modifications to glass and glass-ceramics can be used to introduce greater multifunctionality by enhancing antibacterial properties and to create a new generation of versatile, multifunctional materials for biomedical applications.

2. Bioactive glass and glass-ceramic biomaterials

Since 1969, Hench [21] and their co-workers were largely responsible for the development of bioactive glasses (BGs) and study their bone bonding properties. Later, the work in this field was comprehensively reviewed by Rees Rawlings [22] in 1993, which included a description of the key features and properties of BGs and their glass-ceramics derivatives. In this framework, remarkable developments in the glass and glass-ceramic biomaterials for bone and joint repair and replacement have been made in the last 5 decades. It began with the development of a "bioinert" material, only aiming to minimise the scar tissue formation at the surface of the host tissue. Then, after extensive research it evolved to a BG concept, such as Bioglass® 45S5 with extraordinary interfacial bond properties between implants and bone [23]. Later, a third generation of biomaterials that aiming functional properties such as enhanced cell proliferation and osteogenic properties or even more recently the antibacterial activity, either by inorganic modifications and/or by intelligent design of the glass and

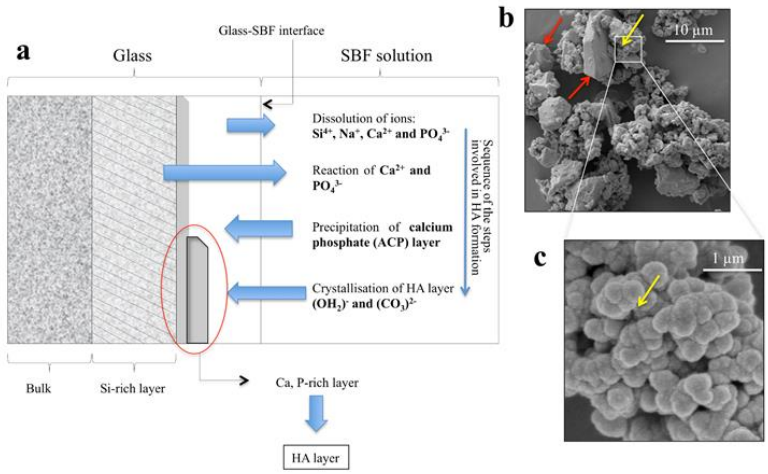
glass-ceramics [1, 16, 18, 21, 24-29]. BGs and in particular the mechanisms responsible for their behaviour in the body have been reviewed extensively by some of the leading figures in the field [30-32]. Their sections on BG science were comprehensive and the subject is therefore only covered briefly here. However, relatively little attention has been paid to the development of antimicrobial glasses, and this is therefore reviewed in far more detail in Section 3, as well as their mechanisms of action in Section 4.

Glass biomaterials can predominantly be fabricated either by the traditional melt-quench or sol-gel processes, where a number of simple compounds are able to mix and solidify as a glass [33-36]. The glass structure is composed of network formers (e.g. Si^{4+}, B^{3+} and P^{3+}), usually silica, which contributes to the network formation containing either intermediate oxides (e.g. Al^{3+}, Zn^{2+}, Mg^{2+}) and/or network modifiers (e.g. Sr^{2+}, Ca^{2+}, Na^{+}). Intermediate oxides, depending on the composition of the glass, may play a network or disrupting function, while network modifiers disrupt the network and produce non-bridging oxygen ions.

A second step of controlled heat treatment is necessary to obtain glass crystallisation forming glass-ceramics [37, 38]. This second heat treatment that leads to crystallisation involves two stages, first a nucleation and then a crystal growth stage, which promote the re-arrangement of the glass structures generating a well-ordered and crystalline structure. Crystallisation can also be a key factor for the fabrication of multifunctional glass-ceramics, modulating theirs resorbability, cytotoxicity and bioactivity [20, 39-42]. However, not all glasses are able to undergo a controlled heat treatment and form glass-ceramics either because they are already too stable or too unstable and difficult to have a controlled heat treatment. Therefore, glasses and

glass-ceramics possess the same building units just arranged in many different patterns, which leads to different final properties. The work in this field was comprehensively revised by Hench *et al.* [21, 43], Rawlings *et al.* [22] and Julian Jones [30].

Silicate glasses, the most used BGs, are well studied to form of a bone-like hydroxyapatite (HA) layer that is fundamental for a strong interfacial bond between the device and bone [21, 23]. The mechanism of bioactivity and bone bonding has been extensively studied *in vitro* (immersion in SBF) and *in vivo*, mainly for 45S5 bioglass® and was discussed elsewhere [44, 45]. Thus, the bonding ability of glass and glass-ceramics relies in the degradation process of the biomaterials and subsequent formation of a HA layer on their surface, which mimics the mineral bone composition, bonding firmly with living bone tissue. Briefly the process follows the succeeding steps, (1) dissolution of ions from the glass into the medium, (2) reaction of dissolved Ca^{2+} and $(PO_4)^{3-}$ from the media and consequent precipitation of amorphous calcium phosphate (ACP) layer, (3) the pH unbalance and increased dissolution of ions supports the growth of ACP, and (4) ACP layer incorporates $(OH)^-$ and $(CO3)^{2-}$ from the media and crystallises as HA layer. Figure 1 shows a schematic of the steps involved in the formation of HA, as well as SEM micrographs of HA structures formed on the surface of glass particles after immersion of SBF.



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132 Figure 1 - (a) Schematic view of the steps involved in the formation of a HA layer. (b)
133 SEM micrographs of BG particles after immersion in SBF showing the apatite-like
134 structures formed at their surface (5000x magnification); (c) detail of apatite-like
135 surface layer (100000x magnification). The red arrows indicate the glass particles and
136 yellow arrows indicate the formed apatite-like structures.

137 The silicate-based glasses and glass-ceramics are commonly associated with slow
138 degradation rates and incomplete conversion to HA. This might result in a mismatch
139 of the degradation rate with the rate of new tissue formation and the presence of long-
140 term unconverted glass and glass-ceramics in the human body [45-48]. More recently,
141 borate- and borosilicate-based glasses have been used with great potential to
142 overcome the drawbacks of the silicate-based glasses [48-50]. Due to their lower
143 chemical durability, borate and borosilicate bioactive (BBGs) glasses present
144 increased biodegradation and more complete conversion to HA. BBGs offer a more
145 controlled dissolution rate that triggers a range of biological responses required for
146 the final biomaterial [51]. Furthermore, boron is associated with bone healing,
147 stimulating bone formation and with the increase in bone resistance to fractures [52-
148 54]. Thereby, the compositional flexibility is at most importance while designing

glasses or glass-ceramics. A number of parameters might influence the design of the BGs. As already been shown a controlled release of ions promotes HA formation leading to an osteointegration, while stimulating osteogenic functions of the surrounding cells [52, 55]. For instance, Maeno *et al* and Santocildes-Romero *et al* [56, 57] showed that ions such as Ca^{2+} and Sr^{2+} influenced both HA deposition and osteogenic behaviour of cells [56, 57]. In fact, Santocildes-Romero *et al.* [57] reported a strong evidence for upregulation of key genes as a mechanism for osteoconductivity or osseoinductivity in both conventional 45S5 and Sr-substituted BGs, which supported previously postulated theories for bioactivity of glasses. Specific trace amount of component ions (e.g. Ag^{+}, Cu^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+} and Zn^{2+}) incorporated and released in a controlled manner can trigger a range of different biological responses, such as bioactivity, osteogenic activity or/and antimicrobial activity [26, 55, 58-60].

Another important issue to consider while designing glass and glass-ceramic formulation are the external local environment generated by the degradation of the biomaterials that might be too harsh for the host tissue. Often, glasses are associated to a certain degree of cytotoxicity, which can potentially affect host cell viability in areas surrounding the medical device [20, 39]. For instance, large increases of pH can induce adverse tissue responses, while the high local osmolarity variations can produce an unbalanced cellular response. Bakry *et al.* [61] showed that some the reported cytotoxic effects of 45S5 bioglass® were associated with the initially acidity of the local environment. In these cases it might be beneficial to use heat treatments in order to induce crystallisation of the glass phase forming a more-stable glass-ceramic, with a better controlled degradation rate [20, 37, 40, 41]. Hurrell-Gillingham *et al.* [41] investigated the effects of devitrification of glass-ionomer cements from SiO_2-

Al_2O_3-P_2O_5-CaO-CaF_2 system onto a glass-ceramic improving its *in vitro* biocompatibility. More recently, Fernandes et al. [20] also demonstrated that a controlled crystallisation of BBGs might be used to improve the biocompatibility of these glass-ceramic systems.

Glass and glass-ceramics are specific ion-containing matrices that latetly are being more often investigated not only for bone repair, but for the prevention and treatment of bone infections. As described above, they have excellent bioactive properties, strongly bonding to bone tissue through complex reactions [21] with good biocompatibility and great osteoinductive and osteoconductive properties [21, 23, 44, 62]. They have been used in the form of particles, porous or dense scaffolds for orthopaedic surgery and dentistry for bone repairing [23, 30, 63]. As a matter of fact, different inorganic modifications have been introduced by several researchers in order to achieve glass and glass-ceramics (Figure 2) endowed with antibacterial properties, resulting either in intrinsic and/or enhanced antibacterial activity. Those biomaterials can be applied through a diversity of final forms depending on their application in the body. As shown in Figure 2a, glasses can either be designed with inorganic species into the bulk glass network or surface modified after glass formation. Moreover, different heating and cooling rates can be used to induce a phase separation, which can create groups of specific ionic components to be released at different rates, tuning biological response. On the other hand, glasses can be submitted to controlled thermal treatments, resulting in to a glass-ceramic (Figure 2b). Different properties can be obtained either by inducing the formation of crystalline phases in a glass matrix or by the formation of a residual glass in to the glass-ceramic matrix providing different releasing profiles, and modulating the bioactive and antimicrobial properties of the glass-ceramics

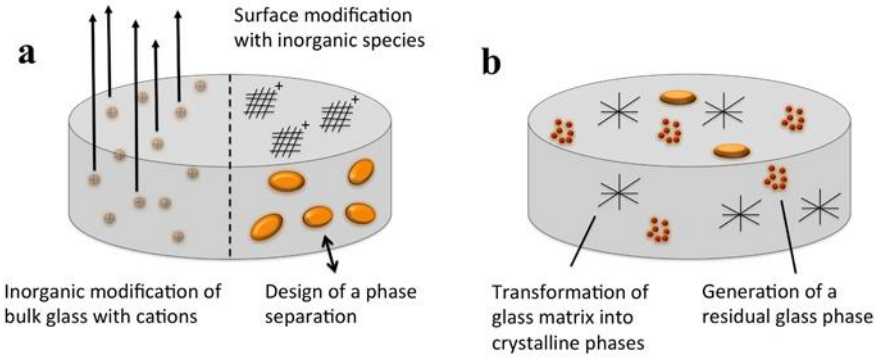

Figure 2 - Potential routes to enhance the antimicrobial properties of a) BGs and b) glass-ceramics via crystallisation of the glass phase.

The following section will review the different glass and glass-ceramics currently proposed to reduce the risk of infection in bone and joint surgery, as well as the potential for these materials to be used to treat deep bone infections themselves.

3. Antimicrobial glass compositions and modifications

While there is undoubtedly a growing clinical need for antimicrobial devices, the regulatory environment makes it increasingly difficult to bring simple drug-device combinations to the market. Major pharmaceutical companies with the potential to make progress are also struggling to justify the development of antibiotics of last resort from an economic standpoint. The Food and Drug Administration (FDA) is approving increasingly fewer antibiotics. Statistical analysis performed by the Center for Disease Dynamics, Economics & Policy (CDDEP) highlighted that only six antibiotics were approved in the period between 2010 and 2014, 10 fewer than in the four-year period between 1983 and 1987. Actually, many of the drugs approved by FDA in the 1980s and 1990s have since been taken off the market for a variety of reasons, including: safety, efficacy or reduced of profitability [64]. Additionally,

antibiotics are not an ideal solution due to challenges in reaching the target organisms, especially when these become associated with a medical device [10, 65, 66]. Local and/or preventive treatments may therefore be a superior approach to deal with bacterial infections. Different methods of loading antibiotic into medical devices are been used for local application of antibiotic although manufactures are focusing their efforts to improve existing active ingredients instead of developing new compounds. Rahaman *et al.* have summarised part of the field, but they limited their review to a narrow range of papers and did not consider more detailed or complex aspects of glass design and structure properties relationships [67].

Glass and glass-ceramic biomaterials have been studied for more than 50 years resulting in the development of hundreds of different formulations. The controlled design or/and modification of the glass and glass-ceramics is the key factor to impart a suitable multifunctionality to the medical device [21, 26, 29, 68-70]. They are design to have suitable osteointegration and have been demonstrating antibacterial activity when specifically assessed [26, 71-74]. Allan *et al.* [71] tested with success the use of 45S5 bioglass® to inhibit several oral bacteria (including *Streptococcus sanguis*, *Streptococcus mutans* and *Actinomyces viscosus*) while repairing periodontal defects. This antibacterial activity has been generally attributed to the release of ions to the reaction media and their effect in the local physiological environment (e.g. pH, osmolarity). Zhang *et al*. [72] have demonstrated that BGs without any special bactericidal components exhibited antibacterial activity towards a large selection of bacteria in a concentration-dependent manner. The authors correlated this activity with the increase of pH and the concentration of alkali ions. In this study the glass S53P4 inhibited the proliferation of all the tested bacteria, e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Enterococcus faecalis*,

Staphylococcus epidermidis. Moya *et al.* [75, 76] studied borosilicate glasses (SiO_2–Na_2O–CaO–B_2O_3 system) with a high content of calcium oxide and found that Ca^{2+} concentration is related with the biocidal activity against Gram-positive, and Gram-negative bacteria. Several other authors also related the antibacterial effect of glass biomaterials with pH and ion concentrations [73, 77, 78]. This type of activity (based on intrinsic antibacterial properties) mainly relies on the degradation of the network and the leaching of species of the surrounding environment. Therefore, it is of most importance to fully understand the mechanisms of glass structure formation and their effect on degradation (Figure 2) to specifically design glasses towards the final application.

Several pathogenic microorganisms (predominantly *Staphylococcus aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*) have been identified at the site of approximately 90% of all implants, and many of these microorganisms present resistance to antibiotics [9, 79, 80]. The critical complications of bacterial contamination are mostly related with the adhesion of bacteria to the medical device, which produce an hydrated extracellular matrix (ECM) generating biofilms [81-83]. These multifaceted structure made from microorganisms and ECM is capable of resisting antibiotics and antibacterial agents, and are at the root of many persistent and chronic bacterial infections [84]. Intrinsic glasses and glass-ceramics have been found to be effective against bacteria sessile communities that are at the root of many persistent and chronic infections. Allan *et al.* [85] showed that 45S5 bioglass® significantly lowered the viability of biofilms of *S. sanguis* when compared with an inert glass control. While, Batalu *et al.* [78] reported that although MgB_2 nano or micropowders did not affected the *S. aureus* biofilm formation, it strongly inhibited *E.*

coli adhesion and viability. Once again, the authors related the activity mainly with pH and the release of boron derivatives.

The intrinsic activity of glass and glass-ceramics is rarely highly specific and uniquely oriented towards prokaryotic cells. However, antibacterial glass and glass-ceramics can be developed by the simple incorporation of specific ions (e.g. Ag^{+}, Ce^{3+}, Cu^{+}, Zn^{2+}, Sr^{2+}) with known antibacterial activity. These ions can either be incorporated into the bulk network of the glasses or at the surface (Figure 2). Within the last few years, a number of glasses and glass-ceramics have been studied specially for their antibacterial properties [59, 77, 86, 87]. The majority of the studies were carried out with silver doped glasses. For instance, Bellantone *et al*. [86] and Ahmed *et al*. [77] demonstrated that silver doped glasses present not only bacteriostatic, but they also caused a rapid bactericidal action against *E. coli*, *P. aeruginosa*, and *S. aureus*. Bellantone *et al*. [86] prepared their silica-based silver containing glass via an acid-catalysed sol-gel route and observed that the dissolution profiles of Ag^{+} from the glasses were consistent with silver accumulation by the bacteria. While, Ahmed *et al*. [77] prepared phosphate-based silver containing glasses by melt-quenching and verified that the increase on antibacterial activity matched the increase in silver content in the glass formulation.

Several ions referred as antibacterial agents were also studied. Brauer *et al.* [88] developed melt-derived BGs of calcium substituted with strontium and tested it as a strontium-releasing injectable bone cement. They demonstrated the antibacterial activity against *S. aureus* and *S. faecalis* for the treatment of osteoporosis-related vertebral compression fracture. Whereas, Mulligan *et al*. [87] used cooper doped glasses to eliminate *S. sanguis* biofilm found in the oral cavity. They prepared

phosphate-based glasses doped with increasing amounts of copper by melt-quenching with the capacity to decrease the viability of *S. sanguis* biofilm. However, after a time period it returned to levels similar to those of the controls. Neel *et al*. [89] also prepared phosphate-based glasses containing copper in the final form of fibres. Those fibres were capable to reduce the number of viable *S. epidermidis* attached to the fibres and in the surrounding environment. Another well know metal, Zinc, was incorporated into silica-nanoparticles prepared by sol-gel showing well defined antimicrobial activity. Halevas *et al*. [90] tested different concentration of incorporated zinc against *S. aureus, Bacillus subtilis, Bacillus cereus, E. coli, P. aeruginosa, Xanthomonas campestris* exhibiting higher activity for higher concentrations. There are other ions, such as cerium and galium that were also tested for antibacterial properties. Goh *et al*. [59] have tested cerium doped glasses for their antibacterial properties. They reported significant improvements regarding the antibacterial activity against *E. coli* of silica-based glasses with 5 mol% of Ce or higher. Valappil *et al*. [91] tested phosphate-based glasses doped with gallium and silver to study their combined action. They showed that the simultaneous release of Ag^{+} and Ga^{3+} from the glass reduced *Porphyromonas gingivalis* biofilm growth with a maximum effect after 168 h.

The composition of the glass is the essence of the antibacterial properties discussed in the paper. Modulating the release rate of ions and, consequently, the osmolarity and pH at the reaction site are at the centre of the reported activity. However, there are other features such as particle size, porosity and morphology that can alter the potency of these biomaterials. It has been found that BGs release rates are directly influenced by surface area. The release of biologically relevant levels of soluble ionic depends on the particle size [92]. Mortazavi *et al*. [93] assessed the antibacterial effect

of BG nanoparticles obtained by sol-gel reporting that the antibacterial activity was caused by a synergetic effect of a high calcium concentration and an alkaline pH, which might have been modulated by the reduction of particle size (20 to 90 nm). Glass composition 58S showed antibacterial activity against *E. coli*, *P. aeruginosa*, and *S. aureus* while 63S exhibit activity only against *E. coli*, *S. aureus* and 72S didn't show any activity.

The level of ions release is directly associated to the roughness of the surface. The higher the roughness, the wider the surface area and therefore higher the release rate. Similarly, higher the porosity of the BGs, higher the surface area, which modulates the release of ions. Some other studies reported the influence of particle size and morphology of glass and glass-ceramics [72, 94]. For instance, Waltimo *et al*. [94] studied SiO_2-Na_2O-CaO-P_2O_5 nano BGs and the influence of their specific surface area in the release of ions and antibacterial activity. They reported that the increase of surface area induces a faster dissolution of alkaline species to the medium, increasing the pH of the medium, and therefore the antibacterial activity. The physico-chemical factors such as roughness and porosity can also influence bacteria adhesion and therefore influencing biofilm formation. The irregularities of the material surfaces normally promote bacterial adhesion, leading to biofilm accumulation whereas a smooth surface do not favour bacterial adhesion and biofilm formation [6].

The bulk materials that exert an antibacterial action in the absence of modifications can generally be described as intrinsically antibacterial. They are designed specifically to present bone integration properties, however, while not being designed as antibacterial materials they can present such activity. Whereas, enhanced glass and glass-ceramics differ from the intrinsic ones because they either have one or more

ionic species intentionally incorporated as antibacterial agents, or are loaded with bactericidal substances or coated with active functional molecules to impart a functional antibacterial activity. Table 1 summarises substituted BGs or glass-ceramics that present antibacterial activity correlating them with their active components.

Table 1 - Examples of BGs or glass-ceramic biomaterials that present antibacterial activity and the correlation with the factors and components responsible for that activity

Active factor	**Glass system**	**Organisms**		**Ref**
		Gram (-)	**Gram (+)**	
Ag^+	SiO_2-CaO-P_2O_5-Ag_2O	*E. coli*	-	[33]
		P. aeruginosa	*S. aureus*	[86]
		E. coli	*S. aureus*	[95]
	P_2O_5-CaO-Na_2O-Ag_2O	*E. coli, P. aeruginosa*	*S. aureus*	[77]
	B_2O_3-Na_2O-P2O_5-Ag_2O	-	*Listeria monocytogenes*	[96]
	SiO_2-Ag (ceramic)	*E. coli*	*S. aureus*	[97]
	Ag_2O-B_2O_3-SiO_2-CaO	*E. coli*	*S. aureus*	[98]
	SiO_2-CaO-P_2O_5-Al_2O_3-Na_2O-K_2O-Ag_2O	*E. coli*	*E. faecalis*	[58]
Ag^+ ; pH	CaO-SiO_2-Ag_2O	*E. coli*	*S. aureus*	[99]
Ag^+ ; Ga^{3+}	CaO-Na_2O-P_2O_5-Ga_2O-Ag_2O	biofilm (*Streptococcus gordonii* and *P. gingivalis*)		[91]
Ag^+ ; Zn^{2+}	Ceramic doped with Ag-Zn	*E. coli*	-	[100]

Ce^{+} ; pH	SiO_2-CaO-P_2O_5-Ce	*E. coli*	-	[59]
Cu^{+}	Na_2O-CaO-P_2O_5-Cu	-	biofilm (*S. sanguis*)	[87]
	Na_2O-CaO-P_2O_5-Cu	-	*S. epidermidis*	[89]
Si^{4+} ; pH	S53P4	*E. coli*	-	[101]
Zn^{2+}	SiO_2-Zn NPs	*E. coli*	*S. aureus*	[102]
	SiO_2-Zn NPs	*E. coli, P. aeruginosa, X. campestris*	*S. aureus, B. subtilis, B. cereus*	[90]
[ions] ; pH	45S5 bioglass®	*E. coli, P. aeruginosa, Actinobacillus actinomycetemcomitans, P. gingivalis, Fusobacterium nucleatum*	*S. sanguis, S. mutans, A. viscosus* and *E. faecalis*	[71, 94, 103]
		biofilms (*S. sanguis*)		[85]
	58S and 63S bioglass®	*E. coli, P. aeruginosa, Salmonella typhi*	*S. aureus*	[93]
	S53P4	*Acinetobacter spp, Haemophilus influenza, Enterobacter aerogenes, M. catarrhalis, E. coli, P. Aeruginosa*	*S. epidermidis, E. faecalis*	[72, 73, 104]

	MgB_2	*E. coli*	*S. aureus*	[78]
	Na_2O-MgO-CaO-B_2O_3-P_2O_3-SiO_2/K_2O/Al_2O_3	*Acinetobacter spp, H. influenza, E. coli, P. aeruginosa*	*E. faecalis*	[72]
	Na_2O-K_2O-MgO-CaO-P_2O_3-SiO_2	*Acinetobacter spp, H. influenza, E. aerogenes, E. coli, P. aeruginosa*	*S. epidermidis, E. faecalis*	[72]
	Na_2O-K_2O-MgO-CaO-B_2O_3-P_2O_3-SiO_2	-	*S. epidermidis*	[73]
	P_2O_5-CaO-Na_2O	*E. coli, P. aeruginosa*	*S. aureus*	[77]
	SiO_2-B_2O_3-Na_2O-MgO/SrO	*P. aeruginosa*	*S. epidermidis*	[26]
	SiO_2-P_2O_5-CaO-Na_2O-SrO	*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*	-	[25]
$[Ca^{2+}]$	SiO_2-B_2O_3-Na_2O-CaO-K_2O-Al_2O_3	*E. coli, P. aeruginosa*	*S. aureus, S. epidermidis and Micrococcus luteus*	[105]
	SiO_2-Na_2O-CaO-P_2O_5-Al_2O_3-Fe_2O/B_2O_3/K_2O/MgO	*E. coli*	*M. luteus, Candida kruse*	[76]
	SiO_2-Na_2O-CaO-B_2O_3/K_2O-Al_2O_3	*E. coli*	-	[75]

$[Ca^{2+}]$; pH	SiO_2-CaO-Na_2O-K_2O-P_2O_5/MgO	-	*S. aureus*	[74]
$[Sr^{2+}]$	SiO-SrO-CaF_2-MgO	-	*S. aureus, E. faecalis*	[88]
pH	CaO-SiO_2	*E. coli*	*S. aureus*	[99]

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350 **4. Mechanisms of antimicrobial activity**

351 Composition is the basis of the properties of glasses and glass-ceramics. It can
352 modulate the rate of ions release and consequently the local osmolarity and pH,
353 influencing the physiological conditions at the surrounding of the medical devices.
354 Therefore, the antibacterial glasses and glass-ceramics is often engaged by their
355 composition and dissolution properties [26, 71, 75, 85].

356 Recently, Echezarreta-López *et al*. [106] compiled from literature a large database on
357 the production of glass biomaterials, bacterial properties and experiments using an
358 artificial intelligence tool, named: neurofuzzy logic technology. They verified that the
359 antibacterial properties of glass and glass-ceramics can be induced by the release of
360 alkaline ions, particularly Ca^{2+} ions, and the increase of the pH of the medium.
361 Briefly, the mechanisms of action are described in three steps: (i) release of ions that
362 increases their (ii) osmolarity and (iii) pH at the reaction site, unbalancing the
363 bacterial intracellular Ca^{2+}, which results in cell membrane depolarisation and their
364 subsequent death. Cabal *et al*. [105] reported that borosilicate glass-ceramics were
365 able to inhibit bacterial growth, minimise bacterial adhesion and prevent biofilm
366 formation by the perturbation of intracellular Ca^{2+} compartmentalisation, causing

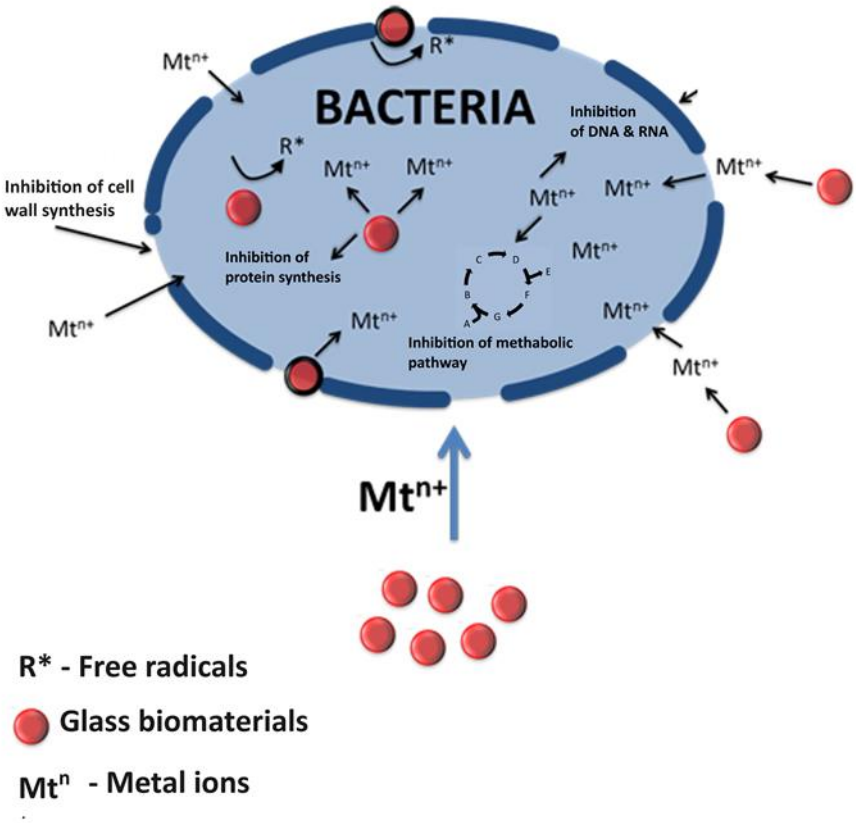
367 cytotoxicity and resulting in either apoptotic or necrotic bacteria cell death. This work
368 tested the borosilicate glasses against five ATCC strains (*S. aureus, S. epidermidis, P.*
369 *aeruginosa, E. coli and M. lutea*) showing a reduction of the viability of bacteria.

370 The antimicrobial activity is dependent on the release rate of ions in an aqueous
371 environment. The lethal effects of those ions on bacterial cells are possibly due to
372 damage to the bacterial cytoplasmic membrane, denaturation of proteins, or damage
373 to the DNA. Ions release from BGs might interact with the cytoplasmic membrane,
374 deregulating the extra- and intracellular enzymes activity. Moreover, the high pH
375 from the ions release also alters the integrity of the cytoplasmic membrane, provoking
376 proteins denaturation. The adjustment of intracellular pH might affect cellular
377 functions, including the essential enzymes for cellular metabolism.

378 However, there are several enhanced glass and glass-ceramics that have their
379 antibacterial activity based in the use of stable metals, such as silver (between many
380 other: Ce^{3+}, Cu^{+}, Zn^{2+}, Sr^{2+}), which are reported to present antibacterial activity [107].
381 BGs are used as carriers for the controlled delivery of active metals. They can be
382 incorporated into the glass structure during fabrication and, while degrade release
383 them at a clinically acceptable rate. In these cases, the antibacterial activity is oriented
384 towards prokaryotic cells and it is usually specific. However, occasionally, they are
385 associated to a certain degree of cytotoxicity towards animal cells [108]. Regarding
386 the use of antibacterial metals, which is frequently active due to their corrosion in the
387 physiological environment, or their leaching to surrounding medium, the high
388 concentration of those ions might also cause local toxicity.

389 A number of studies have been proposing possible mechanisms for antimicrobial
390 properties of those elements, which are associated with disruption of bacteria

function, the unbalance of the electron transport, the binding to DNA or the interaction with the cell components. Even though the exact mechanism of the activity of the metal ions regarding antibacterial action is still unknown it is recognised that it relies on a combination of actions. Silver has been one of the most studied materials, and have been intentionally used in surgeries for its bactericidal properties. It acts by inactivating critical enzymes of the respiratory chain by biding to thiol groups and inducing the formation of hydroxyl radicals promoting oxidative stress [109]. However, other chemical activity, e.g. hydrogen, bonding may also be involved. In this case it inhibits the synthesis of the bacterial cell wall proteins and bacterial RNA and DNA. It has been also reported that it inhibits the metabolic pathway [109, 110]. Therefore, the activity is generally associated to the ionic form of the metal. Moreover, Jung *el al.* [109] demonstrated a higher antibacterial activity against Gram-negative (*E. coli*) than against Gram-positive (*S. aureus*) bacteria. This suggests that the antibacterial activity of the metal ions might be related to the thickness of the peptidoglycan layer of Gram-positive bacteria, which may difficult the action of the silver ions at the bacterial cell membrane. An overview of the hypothesised mechanisms associated with the antibacterial activity of metal particles is presented in Figure 3.



409

410 Figure 3 – Overview of the hypothesised mechanisms associated with the antibacterial
411 activity of metal ions. In the use of silver ions the most pronounced effects is related
412 with cellular metabolic activity (inhibition of respiratory chain and cell pathways), as
413 well as the generation of ROS (oxygen reactive species) and DNA and RNA damage.
414 Diagram was modified from [111].

415

416 **5. In vitro methods for antimicrobial activity evaluation**

417 A variety of screening methods can be used to evaluate *in vitro* antimicrobial activity
418 of glasses and glass-ceramics. The most common are diffusion and dilution methods.
419 The diffusion methods provide qualitative results by categorizing bacteria as
420 susceptible, intermediate or resistant. However, cannot distinguish bacteriostatic and
421 bactericidal. On the other hand, dilution methods are considered quantitative assays

once they determine the minimal inhibitory concentration (MIC) or/and minimum lethal concentration (MLC).

Briefly, the disk-diffusion method consists in agar petri dishes inoculated with standardise inoculum of the testing microorganisms. Then, 6 mm diameter discs containing the desired concentration of the glasses are placed on the agar surface. The petri dishes are incubated under suitable conditions, where the antimicrobial agents diffuse into the agar petri dishes inhibiting the growth of the tested microorganism, and forming measurable growth zones. The disk-diffusion assay is a simple, low-cost allowing to test enormous number of microorganisms and antimicrobial agents with easy access to data analysis.

On the other hand, the broth-dilution method involves preparing two-fold dilutions of the antimicrobial agent in growth medium in separated tubes. Then, each tube is inoculated with the desired inoculum at a standardized concentration. MIC can be determined by the eye. After incubation under suitable conditions for the desired time a sample from the tube can be sub-cultured on the surface of non-selective agar plates to determine the number of surviving cells (CFU/mL) after 24 h incubation, from which MLC can be determined. The broth dilution method is costly and time-consuming, although allows a quantitative evaluation.

Both these methods directly depend on the inoculum size and preparation, type of growth medium and incubation times. Therefore, they have been standardized by Clinical and Laboratory Standard Institute (CLSI) and European Committee in Antibacterial Susceptibility Testing (EUCAST) for testing bacteria, yeast and fungi [112-117]. However, when testing glass materials some modifications of the standardized protocols might be required. Therefore, care should be taken not to

change the basis of microbiology by altering the concentrations of media, inoculum and active glass.

6. Glass and Glass-ceramics in the treatment of infectious bone defects

Bacterial infections associated with contamination of implanted medical devices are a critical complication that often leads to the failure of the implant with significant impact in patient's normal life and wellbeing. Existing treatment for MDAIs often requires local delivery of high doses of antibiotics by carrier materials such as cements, collagen sponges and calcium sulfate, which suffer from several limitations. Multifunctional BGs and glass-ceramics are an interesting alternative system that are not only effective in treating MDAIs but are also have the capacity to stimulate bone regeneration.

BGs, have already shown promising results on the treatment of bone infection in humans without any signs of toxic reactions. In a clinical study, eleven patients with chronic osteomyelitis disease in the lower extremity and the spine were treated with BG S53P4, and nine of them healed without complications [118]. Moreover, the incorporation of various metal ions (i.e. Ag^{+}, $Ga^{2+)}$ in BGs and glass-ceramics demonstrated the improvement of their properties towards the treatment of bone diseases. *In vitro* studies of the incorporation of gallium–silver into phosphate-based glasses showed to be a promising alternative to antibiotics treatments providing a controlled and local delivery of antibacterial gallium and silver ions at the site of infection in the oral cavity [91]. Another study, incorporating Ag-doped BGs into a scaffold for dental applications showed a long-lasting antibacterial activity against *E. coli*, *E. faecalis* with non-cytotoxic effects over dental pulp cells.

The bactericidal effect of the BGs and glass-ceramics is highly dependent on the design, clinical application as well as the system (material) in which it is incorporated. However, the suitable microstructural properties in addition to the bioactive and antibacterial activities of the BGs could lead to its multifunctional potential for healing and regeneration of bone and bone diseases, such as osteomyelitis or periodontitis, and change significantly the currently applied treatments. This new BGs and glass-ceramics could found important medical applications in prostheses, dental devices, coating and composite scaffold. Therefore, the use of these BG systems requires supported by preclinical model studies and creation of strong databases to track and optimise their activity towards the specific disease/condition.

7. Conclusions and future outlook

This review clearly demonstrates that it is possible to use inorganic modification to generate BGs and glass-ceramics into biomaterials that are capable of supressing the growth of pathogenic organisms while at the same time increasing bone tissue regeneration. This strategy offers an alternative (or at least a potent adjuvant) to antibiotics. While a number of classical glass compositions such as S53P4 and 45S5 bioglass® appear to have some antimicrobial activity, there is no doubt that enhanced compositions are far more potent. For example, the addition of Ag^{+}, Zn^{2+}, Cu^{+}, Ce^{3+} and Sr^{2+} all increased the antimicrobial activity of the glasses. While the presence of these specific ions has a direct effect on bacteria, it is important to note that the disruption of the glass is also influenced by small compositional changes (i.e. pH, osmolarity, particle size), which affects the degradation rate of the glass phase. It was noted that these effects were frequently neglected by authors who instead focused solely on the effects of specific ions. Therefore there is a clear need to invest in the

494 structural design of glass and glass-ceramics if truly innovative multifunctional
495 systems are to be developed. These will then provide a predictable, local delivery of
496 antimicrobial ions to the site of infection while promoting bone tissue regeneration.

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